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## L-arginine promotes gut hormone release and reduces food intake in rodents

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**Aims:** To investigate the anorectic effect of L-arginine (L-Arg) in rodents.

**Methods:** We investigated the effects of L-Arg on food intake, and the role of the anorectic gut hormones glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), the G-protein-coupled receptor family C group 6 member A (GPRC6A) and the vagus nerve in mediating these effects in rodents.

**Results:** Oral gavage of L-Arg reduced food intake in rodents, and chronically reduced cumulative food intake in diet-induced obese mice. Lack of the GPRC6A in mice and subdiaphragmatic vagal deafferentation in rats did not influence these anorectic effects. L-Arg stimulated GLP-1 and PYY release *in vitro* and *in vivo*. Pharmacological blockade of GLP-1 and PYY receptors did not influence the anorectic effect of L-Arg. L-Arg-mediated PYY release modulated net ion transport across the gut mucosa. Intracerebroventricular (i.c.v.) and intraperitoneal (i.p.) administration of L-Arg suppressed food intake in rats.

**Conclusions:** L-Arg reduced food intake and stimulated gut hormone release in rodents. The anorectic effect of L-Arg is unlikely to be mediated by GLP-1 and PYY, does not require GPRC6A signalling and is not mediated via the vagus. I.c.v. and i.p. administration of L-Arg suppressed food intake in rats, suggesting that L-Arg may act on the brain to influence food intake. Further work is required to determine the mechanisms by which L-Arg suppresses food intake and its utility in the treatment of obesity.

**Keywords:** animal pharmacology, body composition, energy regulation, GLP-1, obesity therapy

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### Introduction

High protein diets promote satiety and weight loss [1,2], but the exact mechanisms mediating these effects are unclear. Evidence suggests, however, that protein is sensed within the gastrointestinal tract, modulating appetite-regulating pathways [3].

Mechanisms proposed to mediate the effects of high protein diets on food intake, include increased thermogenesis, intestinal gluconeogenesis and changes in gut hormone profiles [4]. Such mechanisms may be instigated by the sensing of the amino acids produced by protein digestion. Rodents adapt their diet to balance amino acid intake [5]. Different types of protein can result in different levels of satiety [6], perhaps reflecting their different amino acid compositions. The recent discovery of promiscuous L-amino acid-sensing G-protein-coupled receptors and their expression in the gastrointestinal tract has

driven speculation that these receptors are involved in amino acid sensing and the regulation of food intake. These receptors include the calcium-sensing receptor (CaSR), the T1R1-T1R3 umami taste receptor complex and the G-protein-coupled receptor family C group 6 member A (GPRC6A) [7].

Amino acids may be sensed in the gut to promote the release of anorectic gut hormones [8]. The aromatic amino acids L-phenylalanine and L-tryptophan induce the release of cholecystokinin (CCK) from isolated I-cells [9]. The anorectic gut hormones glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) (3-36) are released from enteroendocrine L-cells in response to nutrients, including amino acids [10]. Ingestion of a high protein meal alters circulating GLP-1 and PYY levels and promotes their release in both humans and rodents [11,12]. These hormones may act directly on appetite-regulating areas of the brain, but may also have indirect effects. The vagus nerve is one of the major extrinsic nerves, with a key role in the gut–brain axis. Evidence suggests that vagal signalling is involved in the regulation of food intake, and may play a role in gut hormone-mediated satiety. Vagal afferents relay mechanosensory and chemosensory signals from the gut to the nucleus of the solitary tract within the brainstem. In addition, specific gut hormones, in particular CCK, but also ghrelin,

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PYY(3-36) and GLP-1, have been reported to exert their effects on appetite and food intake via vagal afferents [13].

The ability of specific L-amino acids, including L-arginine (L-Arg), to stimulate GLP-1 and PYY release has been studied previously *in vitro* [14,15]. L-Arg, a conditionally essential amino acid, is derived from the diet, endogenous synthesis and protein turnover [16]. L-Arg has a well-characterized effect as a secretagogue promoting insulin release from pancreatic  $\beta$ -cells [17]. Oral L-Arg can also stimulate insulin secretion by promoting GLP-1 release, improving glucose tolerance in mice [18]. L-Arg is a potent agonist of GPRC6A [19], and it has been suggested that GPRC6A activity is necessary for some effects of L-Arg on glucose homeostasis [20]. Furthermore, GPRC6A was required for ornithine-induced GLP-1 release from an *in vitro* model [21], suggesting that GPRC6A may play a role in L-Arg-mediated hormone release. In addition, L-Arg can stimulate growth hormone release from the pituitary, although the mechanism is unclear [22].

L-Arg thus has established effects on hormone release and metabolism. Recent evidence suggests that L-Arg may also be involved in the regulation of food intake [23]; therefore, we investigated the effect of L-Arg on gut hormone release and energy homeostasis in rodents, and explored the potential mechanisms mediating its effects on gut function and food intake.

## Materials and Methods

### Animals

Male C57BL/6 mice, 8–10 weeks (Harlan, Bicester, UK) and male Wistar rats (200–250 g) (Charles River, Margate, UK) were individually housed under controlled temperature (21–23 °C) and humidity on a 12 h light : 12 h darkness cycle. All the animals had *ad libitum* access to standard chow RM1 (SDS, Witham, UK) and water, and were randomized by body weight, unless stated otherwise. The GPRC6A knock-out (GPRC6a-KO) model used in the present studies was generated by the trans-NIH Knock-Out Mouse Project (KOMP) and obtained from the KOMP Repository. The deleted region completely covers the GPRC6a locus [24], and thus this model differs from others previously described [25,26]. The glucose homeostasis phenotype of the knock-out model was assessed before performing feeding studies to address the conflicting reported phenotypes of other GPRC6a KO models [25,27]. All animal procedures were approved and performed under the UK Home Office Animals (Scientific Procedures) Act 1986.

### Feeding Studies

Animals were randomized by body weight and acclimatized to the procedures before all studies. Because of the basic nature of L-Arg solution, L-Arg monohydrochloride (L-Arg-HCl) neutral salt was used in all experiments. For the fasted studies, animals were fasted for 16 h overnight before receiving water or L-Arg-HCl (Sigma, Poole, UK), at doses stated (Table S1, File S1), in the early light phase by either oral gavage (o.g.) or intraperitoneal (i.p.) injection. For *ad libitum*-fed animal studies, the same administration procedure was used without

fasting in either the early light phase or at the onset dark phase. For the study investigating the role of gut hormones in mediating the effect of L-Arg on food intake, fasted or *ad libitum*-fed mice were given simultaneous i.p. administration of 400 nmol/kg exendin 9-39 (GLP-1R antagonist) and BIIE0246 ( $Y_2$  receptor antagonist) at 5.26  $\mu$ mol/kg 15 min before the o.g. administration of water or 24 mmol/kg L-Arg. Animals were returned to their cages, with pre-weighed amounts of standard chow diet and food intake measured 1, 2, 4, 8 and 24 h after administration. The GPRC6a-KO mice feeding studies used a crossover design, in which GPRC6a-KO and wild-type (wt) mice received both control and L-Arg treatments in random order on separate occasions separated by at least 3 days; accordingly, food intake was compared and analysed using a paired analysis approach. A summary of all feeding studies, including doses, species and time of day of the study, is provided in Table S1, File S1.

### Energy Expenditure Studies

Mice were individually housed in a 24-chamber open-circuit comprehensive laboratory animal monitoring system (CLAMS; Columbus Instruments, OH, USA) and acclimatized for 24 h to generate stable reference data. They were then fasted for 16 h overnight and subsequently received water or 24 mmol/kg L-Arg o.g. (n = 12/group) at 09:00 hours (early light phase). The mice continued to be fasted for the subsequent 8 h, to examine the effects of L-Arg on energy expenditure, independent of effects on food intake, before food was returned at 17:00 hours. Metabolic variables ( $VO_2$  and  $VCO_2$ ) and the respiratory exchange ratio (RER) were measured every 24 min for 24 h after treatment administration, and values normalized to body weight [28].

### Chronic Feeding Studies in Mice

Male mice, aged 6–8 weeks, were group-housed (five per cage) with *ad libitum* access to water and a 60% high fat diet (Research Diets, New Brunswick, NJ, USA) for 8 weeks. The mice were then individually housed and given 1 week to acclimatize before the study started, remaining on the high fat diet. Mice were given water or 16 mmol/kg L-Arg o.g. (n = 9 per group) twice daily throughout the dark phase at 19:00 hours and then 01:00 hours for five nights. Body weight and food intake were measured daily at the beginning of the dark phase and at 1 h after the first daily gavage.

### Subdiaphragmatic Vagal Deafferentation Surgery in Rats

Subdiaphragmatic vagal deafferentation (SDA) was carried out in rats, as previously described [29,30], as it results in more accurate deafferentation and lower morbidity than in mice. The effect of oral administration of water or 16 mmol/kg L-Arg (n = 9–10, crossover) on food intake was then studied in overnight fasted rats during the early light phase.

### Murine Colonic Crypt Isolation and Hormone Secretion Assays

Primary mice colonic crypt isolation and secretion studies were performed using an adaptation of an established method

previously described [31,32]. Gut hormone secretion was expressed as a fraction of the total peptide (secreted plus intracellular) measured in each well over 2 h.

### *In Vitro* Mucosal Studies

Ileal or colonic mucosa from wt male mice (>15 weeks old), was voltage-clamped at 0 mV in Ussing chambers, as described previously [33]. Vectorial ion transport was measured continuously as short-circuit current ( $I_{sc}$ ;  $\mu A/cm^2$ ) and provided an acute readout for endogenous PYY release. Once stable  $I_{sc}$  levels were achieved, vehicle or the  $Y_1$  receptor ( $Y_1R$ ) antagonist BIBO3304 (BIBO; 300 nM) and L- or D-Arg were added to the apical or basolateral reservoirs bathing mucosae. L-Arg (1 mM) responses were measured 15–20 min after vasoactive intestinal peptide (10 nM), an optimum secretory stimulus for revealing subsequent  $G_{ai}$ -coupled epithelial responses [33]. Epithelial  $Y$  agonism results from  $G_{ai}$ -coupled attenuation of cAMP levels, with consequent sustained decreases in  $Cl^-$  ion secretion and  $I_{sc}$  levels [34], thus PYY (10 nM) was added after L-Arg as a control.

### *In Vivo* Gut Hormone Studies

Rats were fasted overnight, before receiving o.g. of either water or 16 mmol/kg L-Arg ( $n=6-8$ ) in the early light phase. They were immediately returned to their cages, killed by decapitation at 30 or 90 min after administration, and plasma samples were collected as previously described [29].

### Intra-ileal Administration Studies

Intra-ileal administration procedures were performed in anaesthetized rodents as previously described [32]. Rats received an injection of either saline or 1 M L-Arg ( $n=4-5$ ) in a volume of 2.5 ml into the upper ileum; blood samples were collected at -15, 0, 15, 30, 45 and 60 min post-administration via the jugular cannula. Mice were injected with either saline or 1 M L-Arg ( $n=4-5$ ) in a volume of 500  $\mu l$  into the upper ileum, and were killed 30 min after administration and blood was collected.

### Gut Hormone Radioimmunoassay

We measured GLP-1 and PYY using previously established in-house specific and sensitive radioimmunoassays [35,36]. The GLP-1 antibody has 100% cross-reactivity with all amidated forms of GLP-1, but does not cross-react with glycine extended forms. The PYY antibody has 100% cross-reactivity with PYY(1-36) and PYY(3-36). The intra-assay coefficients of variation for GLP-1 and PYY assays were 8.7 and 6.0%, respectively.

### Intracerebroventricular Cannulation and Administration

The intracerebroventricular (i.c.v.) injections were carried out as previously described [37]. The rats recovered from surgery for 7 days before being injected with 5  $\mu l$  of either vehicle saline control or 4  $\mu M$  L-Arg ( $n=8-9$ ) over 1 min using a 28-gauge stainless steel injector in the early light phase.

### Statistical Analyses

Acute feeding studies data and area under the curve data are expressed as mean  $\pm$  standard error of the mean (s.e.m.) and were analysed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. CLAMS data were analysed using two-way ANOVA and a Bonferroni *post hoc* test, chronic and SDA feeding study data using multiple Student's *t*-test, GPRC6a-KO feeding data using two-way ANOVA with Sidak's *post hoc* analysis, and *in vitro* and *in vivo* gut hormone data using one-way ANOVA with Dunnett's and two-way ANOVA with Bonferroni's *post hoc* test, respectively. Mucosal data measuring the maximum changes in  $I_{sc}$  are expressed as mean  $\pm$  s.e.m. per unit area ( $cm^2$ ), and single comparisons performed using Student's unpaired *t*-test. GRAPHPAD PRISM software (Prism 6.03, GraphPad Software Inc, CA, USA) was used for all analyses.

## Results

### Effect of Oral L-Arginine on Food Intake, Body Weight and Energy Expenditure in Rodents

Oral administration of L-Arg significantly reduced food intake in both rats and mice (Figure 1). In rats, oral administration of L-Arg suppressed food intake in fasted rats in the early light phase (Figure 1A) and in *ad libitum*-fed rats in the early dark phase (Figure 1B). Similarly, oral administration of L-Arg in fasted mice reduced food intake in a dose-dependent manner (Figure 1C). Oral administration of L-Arg in *ad libitum*-fed mice significantly reduced cumulative food intake 24 h after administration in the light and dark phases (Figure 1D and E). These anorectic effects were not secondary to abnormal behavioural side effects in rats (Figure S1, File S1).

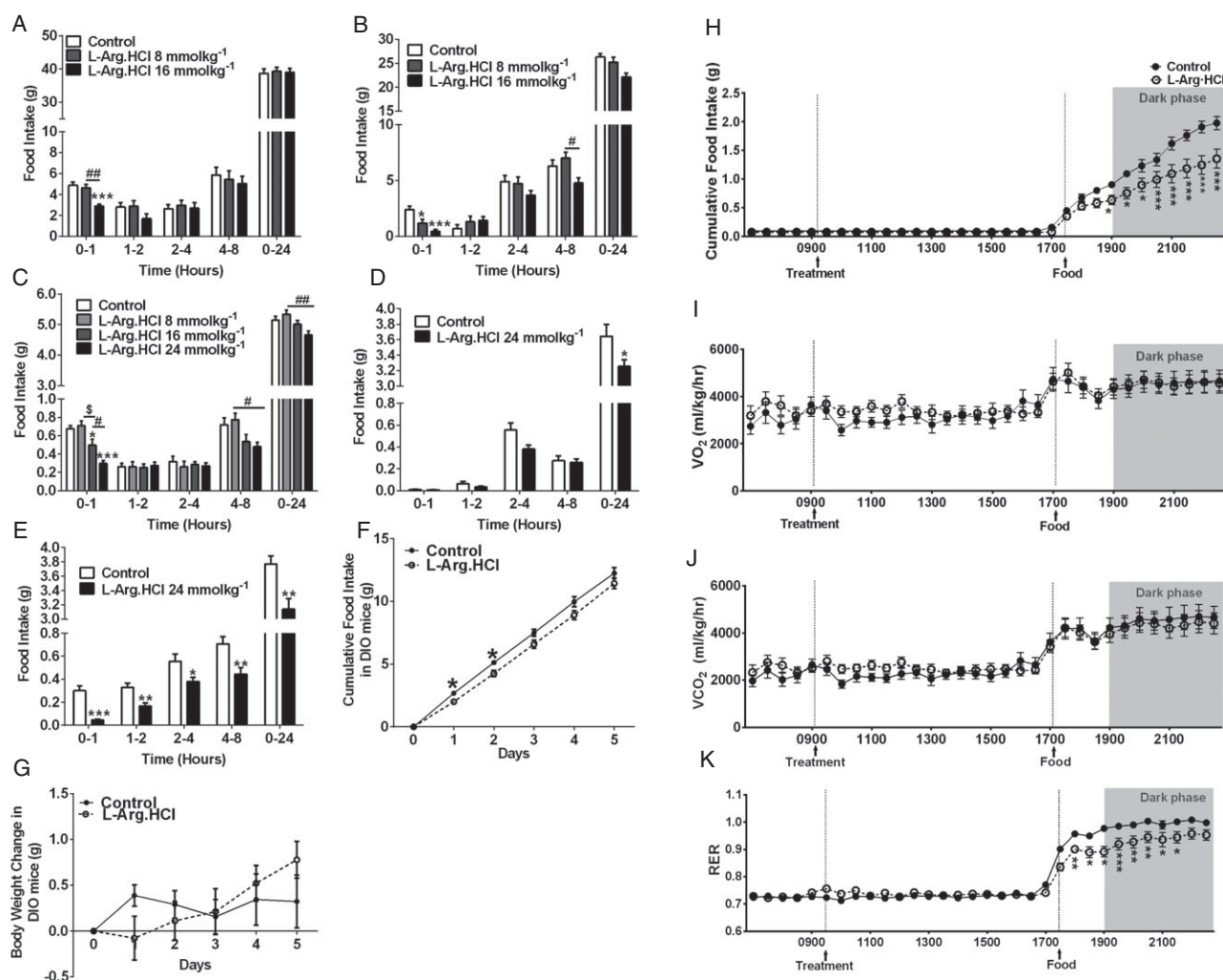
After observing that L-Arg administration could result in a sustained reduction in food intake in rodents, we investigated whether this anorectic effect could be sustained chronically and reduce body weight in a diet-induced obese (DIO) mouse model, a commonly used model of obesity. Repeated L-Arg administration significantly reduced food intake on day 1 and day 2 in DIO mice compared with vehicle-treated mice (Figure 1F), although this effect was insufficient to significantly change body weight over the time period studied (Figure 1G).

To investigate the effect of L-Arg on energy expenditure, the mice were placed in CLAMS metabolic cages. Oral administration of 24 mmol/kg L-Arg had no significant effect on  $VO_2$ ,  $VCO_2$  or RER in mice during the 8 h after administration. Interestingly, oral administration of 24 mmol/kg L-Arg still reduced food intake when food was returned 8 h after administration in mice placed in CLAMS cages, showing a delayed and sustained anorectic effect (Figure 1H). Returning food did not significantly alter  $VO_2$  or  $VCO_2$  between the treatment groups (Figure 1I and J); however, the RER was significantly lower in L-Arg-treated mice, after the return of food (Figure 1K).

### GPRC6A is Not Required for the Anorectic Effect of L-Arginine in Mice

Oral administration of 16 or 24 mmol/kg L-Arg significantly reduced food intake in both wt and GPRC6a-KO mice to a





**Figure 1.** Effect of oral administration of L-arginine (L-Arg) on food intake, body weight and energy expenditure in rodents. Effect of oral gavage (o.g.) of control (water) and 8 and 16 mmol/kg L-Arg on food intake in male rats (A) after an overnight fast [ $n = 9-10$ ;  $\#P < 0.01$  vs. L-Arg (8 mmol/kg),  $***P < 0.001$  vs. water control] and (B) *ad libitum* fed at the beginning of dark phase ( $n = 12-16$ ;  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  vs. water control,  $\#P < 0.05$  vs. 8 mmol/kg L-Arg) at 0–1, 1–2, 2–4, 4–8 and 0–24 h after administration. (C) Effect of o.g. of control (water) and 8, 16 and 24 mmol/kg L-Arg on food intake in male mice after an overnight fast at 0–1, 1–2, 2–4, 4–8 and 0–24 h after administration ( $n = 8-9$ ;  $*P < 0.05$ ,  $***P < 0.001$  vs. water control;  $\#P < 0.05$ ,  $\#P < 0.01$  vs. 8 mmol/kg L-Arg;  $\$P < 0.05$  vs. 16 mmol/kg L-Arg). Effect of o.g. of control (water) or 24 mmol/kg L-Arg in *ad libitum*-fed mice during the early light phase (D) ( $n = 10$  per group), and (E) early dark phase ( $n = 10$  per group) at 0–1, 1–2, 2–4, 4–8, and 0–24 h after administration ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  vs. control). Effect of repeated o.g. administration of L-Arg on food intake (F) and body weight (G) in diet-induced obese (DIO) mice. Effect of three times daily o.g. administration of control (water; black circles, solid line) or 16 mmol/kg L-Arg (white circles, dotted line) on cumulative food intake and body weight change in DIO mice during a period of 5 days ( $n = 9$  per group;  $*P < 0.05$ ,  $***P < 0.001$  vs. vehicle). Effect of o.g. administration of control (water; black circles, solid line) or 24 mmol/kg L-Arg (white circles, dotted line) on cumulative food intake (H), O<sub>2</sub> consumption (I) and CO<sub>2</sub> production (J) and respiratory exchange ratio (RER) (K) in mice injected at early light phase and placed in comprehensive laboratory animal monitoring system cages. The o.g. was performed at 09:00 hours and food was returned at 17:00 hours, as indicated by the dotted line. Recordings were taken over a period of 24 h and at subsequent 24-min intervals after administration. The shaded areas represent the dark phase from 19:00 hours ( $n = 12$  per group;  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  vs. water control). All data are presented as mean  $\pm$  standard error of the mean.

similar magnitude 0–1 h after administration, suggesting that GPRC6A is not necessary for the anorectic effect of L-Arg (Figure 2A and B). Oral L-Arg also improved glucose tolerance in both wt and GPRC6a-KO mice (Figure S2, File S1).

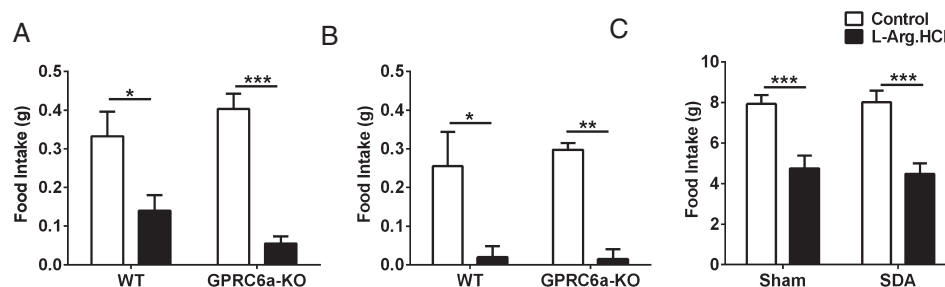
### Anorectic Effect of L-Arginine is Not Mediated via the Vagus

Oral gavage of 16 mmol/kg L-Arg significantly reduced food intake in both sham-operated and SDA-operated rats 0–1 h

after administration, suggesting the vagus is not necessary for the anorectic effect of L-Arg (Figure 2C).

### Effect of L-Arginine on Gut Hormone Release

L-Arginine stimulated GLP-1 and PYY release from murine primary colonic L-cells (Figure 3A and B). Exposure to 100 mM L-Arg for 2 h stimulated PYY release from colonic cultures isolated from GPRC6a-KO, although the GLP-1 response to



**Figure 2.** Effect of L-arginine (L-Arg) on food intake in G-protein-coupled receptor family C group 6 member A (GPRC6a)-KO mice and rats after subdiaphragmatic vagal deafferentation (SDA) surgery. Effect of oral gavage (o.g.) administration of (A) control (water) or 16 mmol/kg L-Arg, and (B) control (water) or 24 mmol/kg L-Arg on 0–1-h food intake in wildtype and GPRC6a-KO mice with *ad libitum* access to food injected at the beginning of dark phase [ $*P < 0.05$ ,  $**P < 0.01$  vs. water control ( $n = 4$ , crossover)]. (C) Effect of o.g. of control (water) and 16 mmol/kg L-Arg on 0–1-h food intake in male rats that underwent sham or SDA surgery ( $n = 9–10$ , crossover;  $***P < 0.001$  vs. control). All data are presented as mean  $\pm$  standard error of the mean.

L-Arg appeared to be attenuated in GPRC6a-KO compared with wt mice (Figure S3, File S1). In keeping with observations *in vitro*, oral administration of 16 mmol/kg L-Arg elevated plasma GLP-1 and PYY release in rats. Plasma GLP-1 levels were significantly elevated at 30 and 90 min after administration compared with control (Figure 3C). PYY levels were significantly elevated at 30 min after administration (Figure 3D). Furthermore, direct upper ileal administration of 1 M L-Arg elevated plasma GLP-1 ( $P < 0.05$ ) and PYY ( $P = 0.07$ ) levels in anaesthetized mice and rats (Figure 3E and F).

### Effects of L-Arginine-Stimulated Gut Hormone Release

To investigate whether the anorectic effect of L-Arg is mediated by increases in gut hormone levels, GLP-1 and  $Y_2$  receptors were simultaneously antagonized in mice receiving 24 mmol/kg L-Arg o.g. The GLP-1 receptor antagonist exendin9-39 and the  $Y_2$  receptor antagonist BIIE0246 were administered i.p. at doses established to block the anorectic effects of exogenous exendin-4 and PYY, respectively (Figure S4, File S1). O.g. of 24 mmol/kg L-Arg significantly reduced food intake, both in fasted mice in the early light phase and fed mice in the early dark phase, whether they were co-administered saline control or a mixture of 5.26  $\mu$ mol/kg BIIE0246 and 400 nmol/kg exendin9-39 (Figure 4A and B).

In line with the hormone release measured *in vitro* and *in vivo*, mucosal  $I_{sc}$  measurements showed that L-Arg (1 mM) altered ion transport acutely within 15–30 min, while D-Arg was inactive (Figure 3C–G). Apical treatment of ileal and colonic mucosae with L-Arg increased  $I_{sc}$  initially (potentially a GLP-1-mediated effect). It then decreased  $I_{sc}$  more slowly and, importantly, this response component was  $Y_1$ R-dependent and therefore most likely PYY-mediated (Figure 4C–I).

### Effect of Central and Intraperitoneal Administration of L-Arginine on Food Intake in Rodents

The i.p. administration of 4 and 8 mmol/kg L-Arg significantly reduced food intake in rats 0–1 h after administration compared with saline controls (Figure 5A). Similarly, in mice, 12 mmol/kg L-Arg significantly reduced food intake 0–1 h after administration. Food intake was significantly lower in mice

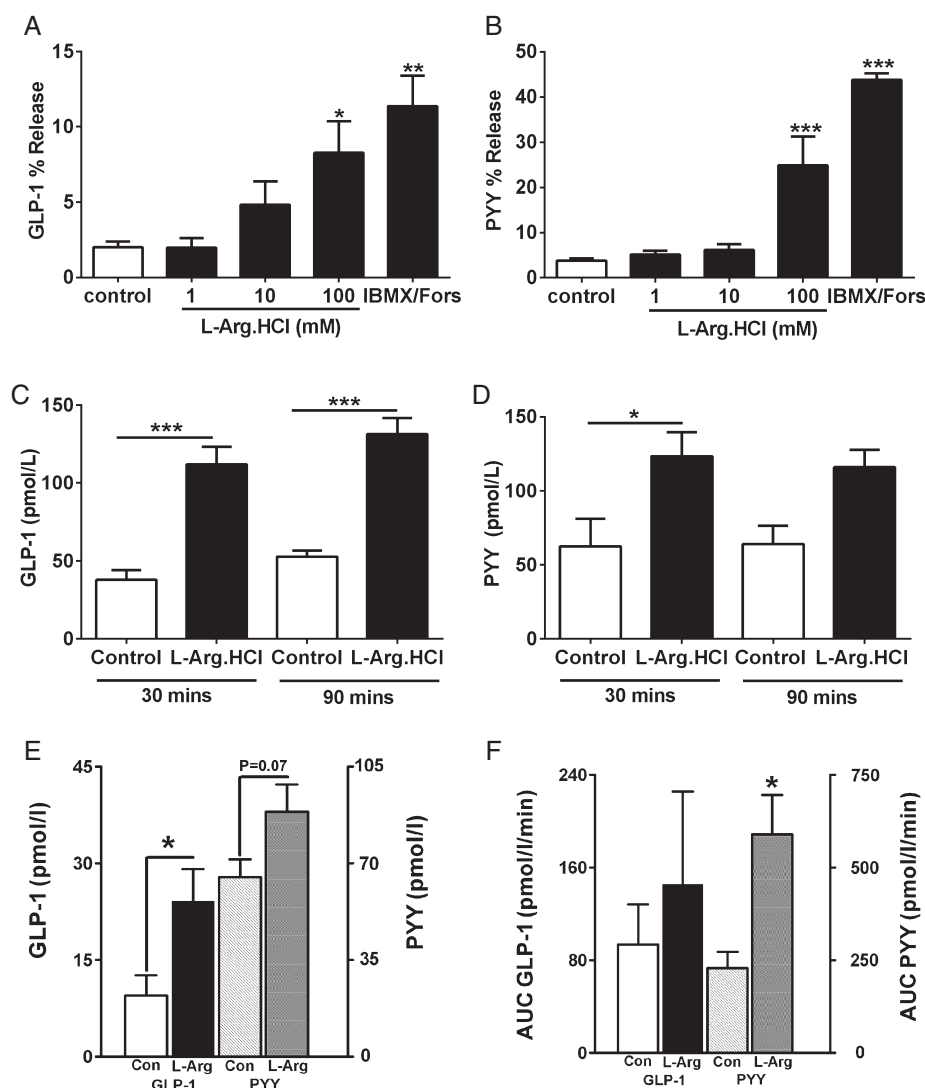
treated with 12 mmol/kg L-Arg 4–8 h after administration. The cumulative food intake at 8 h post-administration was significantly lower in both 8 and 12 mmol/kg L-Arg groups compared with the saline control (Figure 5B). Furthermore, i.c.v. administration of L-Arg significantly reduced food intake in rats at 0–1 h post-administration only, but had no effect on 0–24 h cumulative food intake (Figure 5C).

## Discussion

We investigated the anorectic properties of L-Arg in rodents and the potential mechanisms by which these effects are mediated. Our data show that L-Arg reduces food intake in both mice and rats without causing behavioural side effects, but does not affect energy expenditure in mice at the dose investigated. Repeated L-Arg administration reduced cumulative food intake on days 1 and 2 in DIO mice, but without significant effect on body weight over the time studied. The anorectic effects of L-Arg were not dependent on the amino acid sensing receptor GPRC6A or on vagal signalling. L-Arg significantly stimulated GLP-1 and PYY release acutely *in vitro* and *in vivo*; however, its anorectic effects appear unlikely to be mediated by changes in these gut hormones.

The anorectic effect of L-Arg has been previously shown in a rat model. Jordi et al. [23] showed a significant reduction in food intake after o.g. of 6.7 mmol/kg L-Arg in rats. In our initial dose finding studies, doses of 4 and 6 mmol/kg of the non-salt L-Arg solution suppressed food intake in rats; however, equivalent doses of the L-Arg-HCl salt had no effect on food intake and a dose of 8 mmol/kg or higher was required to significantly reduce food intake in rats. This suggests that the non-salt L-Arg may influence food intake at least in part because of its basicity. To avoid any possible pH-dependent effect, the neutral L-Arg-HCl salt was used in all of our reported experiments. While it is possible that L-Arg-HCl solution has non-specific osmotic effects on food intake, pilot studies using isomolar concentrations of sodium chloride showed no effect of this concentration of sodium chloride on food intake, and found that L-Arg reduced food intake compared with iso-osmotic controls (data not shown).

We found that acute administration of L-Arg had no significant effect on  $VO_2$  and  $VCO_2$  in mice; however, the study

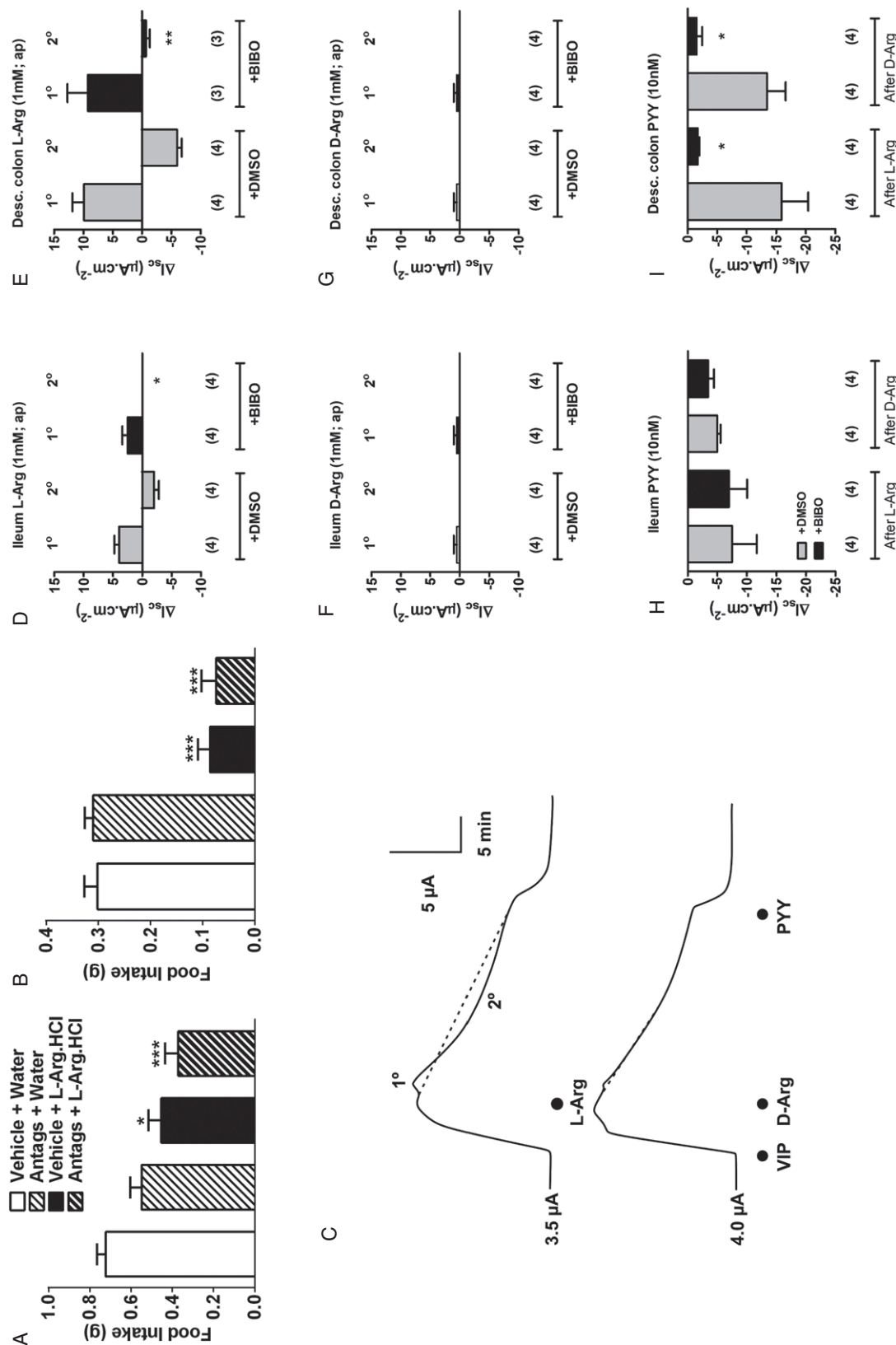


**Figure 3.** Effect of L-arginine (L-Arg) on gut hormone release. Effect of L-Arg on (A) glucagon-like peptide-1 (GLP-1) and (B) peptide YY (PYY) release from primary mice colonic L-cells incubated with 1, 10 and 100 mM L-Arg and IBMX-forskolin mix (10  $\mu$ M, each) for 2 h. The release is shown as percentages of total hormone contained for each well in the experiment ( $n = 9$  plates from nine mice;  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  vs. control). Data presented as mean  $\pm$  standard error of the mean (s.e.m.). Effect of oral gavage (o.g.) administration of control (water) and 16 mmol/kg L-Arg on (C) GLP-1, and (D) PYY in overnight fasted male rats at 30 and 90 min after administration ( $n = 6-8$ ;  $*P < 0.05$ ,  $***P < 0.001$  vs. water control,  $##P < 0.01$  vs. 12 mmol/kg L-Arg). Data are presented as mean  $\pm$  s.e.m. Effect of intra-ileal administration of saline and 1 M L-Arg on plasma GLP-1 and PYY concentrations in overnight fasted (E) anaesthetized mice and (F) rats. Blood samples were taken from mice at 30 min, and from rats at 0, 15, 30, 45 and 60 min after administration ( $n = 4-5$  per group;  $*P < 0.05$  vs. control). Mice results are expressed as mean  $\pm$  s.e.m. Rat results are expressed as area under the curve (AUC) mean  $\pm$  s.e.m.

showed that o.g. of 24 mmol/kg L-Arg significantly reduced food intake in mice when food was returned 8 h later. This shows a prolonged anorectic effect that is not necessarily observable if food is returned immediately. It also suggests L-Arg can have long-term effects on food intake when food is not immediately available after administration, which may be exploitable by weight-loss promoting agents. Furthermore, RER was significantly lower in the L-Arg-treated cohort once food was returned. This effect probably reflects the significantly lower food intake in the L-Arg-treated rodents. Different experimental conditions may alter the pharmacokinetic profile of L-Arg, and further work is required to determine whether such

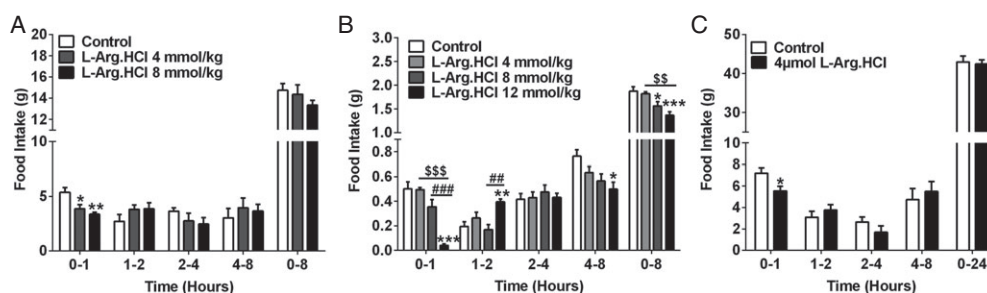
long-term effects occur in other contexts. It is also possible that the effects observed reflect the action of L-Arg metabolites or other molecules of which it is a precursor, such as nitric oxide and glutamate; however, simple availability of L-Arg does not regulate the levels of many of these downstream agents.

Chronic L-Arg supplementation in mice on a low protein diet has been reported to reduce epididymal fat, while increasing food intake [38]. L-Arg may facilitate increased protein synthesis in animals deficient in protein, but its effects might be expected to be very different in rodents with a normal protein intake. We found repeated administration of L-Arg reduced cumulative food intake but did not significantly influence



**Figure 4.** Effect of L-arginine (L-Arg)-mediated gut hormone release on food intake and gut function. Effect of intraperitoneal administration of a mixture of 400 nmol/kg exendin 9-39 and 5.26  $\mu$ mol/kg BII0246 on the anorectic effect of oral gavage of 24 mmol/kg L-Arg in (A) fasted mice during early light phase (n = 10) and (B) *ad libitum*-fed mice during dark phase (n = 10) in the 0–1 h period after administration (\* $P$  < 0.05, \*\*\* $P$  < 0.001 vs. vehicle control group). Data are presented as mean  $\pm$  standard error of the mean (s.e.m.). (C) Representative recordings from mouse colon mucosa showing a biphasic  $I_{sc}$  change to apical L-Arg (1 mM, upper) compared with minor effects to apical D-Arg (1 mM, lower). Basolateral vasoactive intestinal peptide (10 nM) pre-treatment increased  $I_{sc}$ , and subsequent control PYY (10 nM, basolateral) anti-secretory responses are evident. Basal  $I_{sc}$  values (in  $\mu A$ ) are shown to the left of each trace (exposed mucosal area, 0.14  $cm^2$ ). Responses to apical L-Arg, D-Arg and control PYY responses in ileum (D, F and H) and colon (E, G and I) are shown after either vehicle (+DMSO, 0.03%) or  $Y_1$  R antagonist BIBO3304 (+BIBO, 300 nM). Responses are the mean  $\pm$  s.e.m. from observation numbers in parenthesis. Only L-Arg  $2^\circ$   $I_{sc}$  reductions were sensitive to BIBO treatment in (D) ileum and (E) colon mucosae. Note PYY responses in the ileum (H) are attributable to  $Y_2$  signalling (and thus are not significantly reduced by BIBO) while  $Y_1$  R signalling predominates in the mouse colon and is BIBO-sensitive (I). \* $P$  < 0.05, \*\*\* $P$  < 0.001. All data are presented as mean  $\pm$  s.e.m.





**Figure 5.** Effect of intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) administration of L-arginine (L-Arg) on food intake in rodents. Effect of i.p. administration of (A) control (saline), 4, and 8 mmol/kg L-Arg on food intake in fasted male rats ( $n = 8-9$ ;  $*P < 0.05$ ,  $**P < 0.01$  vs. control) and (B) control (saline), 4, 8 and 12 mmol/kg L-Arg in fasted male mice ( $n = 7-9$ ;  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  vs. control;  $$$$P < 0.01$ ,  $$$$$P < 0.001$  vs. 4 mmol/kg L-Arg;  $##P < 0.01$ ,  $###P < 0.001$  vs. 8 mmol/kg L-Arg) at 0-1, 1-2, 2-4, 4-8 and 0-8 h after administration during early light phase. (C) Effect of i.c.v. administration of control (saline) and 4  $\mu$ M L-Arg on food intake in male rats following an overnight fast at 0-1, 1-2, 2-4, 4-8 and 0-24 h after administration ( $n = 8-9$ ;  $*p < 0.05$  vs. control). All data are presented as mean  $\pm$  s.e.m.

weight gain in DIO mice. A longer period of administration and perhaps a higher dose might result in a significant effect on body weight. Our data do not exclude the possibility of small changes to body weight that we were unable to detect in response to chronic L-Arg administration. It is also possible that L-Arg promotes the absorption of other nutrients or causes small decreases in energy expenditure that were not detectable by the CLAMS, which would explain the lack of difference in body weight. Further work is required to establish the chronic effects of L-Arg administration on energy homeostasis.

L-Arg can influence hormone release from other endocrine tissues including the pancreas [17] and the pituitary gland [22]. We therefore investigated the role of anorectic gut hormones in mediating the effect of L-Arg on food intake. L-Arg stimulated the release of GLP-1 and PYY *in vitro* and *in vivo*. Our data suggested that the anorectic effects of L-Arg are not mediated via gut hormone release, although a potential role for other gut hormones cannot be excluded. Mucosal studies, however, complemented the hormonal release we observed and indicated that L-Arg caused acute endogenous PYY release with consequent rapid inhibition of local epithelial ion transport that was  $Y_1$ -mediated. This mechanism is similar to that described for other amino acids acting via the CaSR in mouse colon [39]. PYY and GLP-1 inhibit gastric emptying, the former most likely acting via  $Y_1$  and  $Y_2$  receptors [40]; PYY(1-36) binds to both receptors with similar affinity, while the truncated form which reduces appetite has higher affinity for the  $Y_2$  receptor. Furthermore, L-Arg reduces gastric emptying in humans, apparently via changes in basal levels of nitric oxide [41,42]. In addition, both GLP-1 and PYY inhibit gastric motility in rats [43,44]. Increased plasma GLP-1 and PYY after L-Arg administration may slow gastric emptying acutely, but our evidence suggests that these rapid changes in hormone levels may not be responsible for the subsequent reduction in food intake, which was not blocked by antagonising either GLP-1 or PYY- $Y_2$  receptors. Both PYY(1-36) and PYY(3-36), however, probably influence upper gastrointestinal transit, including gastric emptying, in mice [40], and it is therefore possible that the effects of L-Arg on PYY(1-36) release alter gastric emptying via the  $Y_1$  receptor sufficiently to account for some of the observed anorectic effect in mice.

Basic amino acids including L-Arg are potent activators of GPRC6A [19], which is highly expressed in the gastrointestinal tract and is involved in a number of important physiological pathways [45]. The effects of L-Arg were examined in mice lacking the GPRC6A at both 24 mmol/kg, which was previously shown to reduce food intake in mice, and at a lower dose of 16 mmol/kg, in case the effects of higher doses were mediated by different, perhaps non-physiological mechanisms; however, at both doses, oral administration of L-Arg significantly reduced food intake in GPRC6a-KO mice. Previously, small interfering RNA-induced depletion of endogenous GPRC6A has been shown to abolish L-ornithine-stimulated GLP-1 release from GLUTag cells [21]. GPRC6A ablation did not appear to block L-Arg-induced GLP-1 and PYY release from a primary cultured murine colonic epithelium, although the effect on GLP-1 release was attenuated. These data suggest that GPRC6A is not necessary for the anorectic effects of L-Arg, and that it plays at most a minor role in its effects on gut hormone release. L-Arg also activates both T1R1-T1R3 and CaSR receptors, albeit to a lesser extent than GPRC6A [7]. The involvement of these receptors cannot be ruled out. L-Arg-induced GLP-1 and PYY release from isolated rat small intestinal loops was attenuated by a CaSR antagonist, suggesting it is in part mediated by CaSR [15].

Other mechanisms may be involved in mediating the effects of L-Arg. Evidence suggests L-Arg stimulates the release of insulin from pancreatic  $\beta$ -cells by causing membrane depolarization, and that this effect is not mediated by calcium or ATP-sensitive potassium channels, but as a consequence of electrogenic transport of L-Arg into the  $\beta$ -cell via specific amino acid transporters [46]. Amino acid transporter systems may also be involved in amino acid sensing in the gut [14]. *In vitro* studies suggest that L-cells exhibit action potential-driven calcium influx in response to nutrients including amino acids, leading to acute hormone release. The sodium-coupled neutral amino acid transporter 2 (SNAT2) has been implicated in nutrient sensing and gut hormone release. SNAT2 acts as a secondary active transporter by coupling the transfer of amino acids against their concentration gradient to the simultaneous inward movement of sodium ions down its electrochemical gradient. This sodium-dependent transport mechanism has

been shown to increase intracellular calcium levels and consequently to stimulate the release of gut hormones [47]. Notably, L-glutamine has been shown to stimulate GLP-1 release from intestinal L-cells via a SNAT2-mediated mechanism [14]. In addition, the CaSR has been shown to mediate the pharmacological effects of specific amino acids on gut hormone release from cell lines and *ex-vivo* tissue [15,48].

There is evidence that the vagus nerve responds to nutrient load and is involved in protein-induced satiety [3]. Proteins and amino acids activate neurons within the nucleus of the solitary tract via visceral vagus mediated signals. In addition, GLP-1, Y<sub>2</sub> and CCK receptors are expressed on vagal afferents which are proposed to play a key role in gut-brain mediated responses in satiety and food intake regulation [49]. We examined the effect of L-Arg on food intake in rats that had undergone SDA surgery and observed no difference on the effects of L-Arg on food intake, in accord with previous work suggesting the anorectic effect of L-Arg is not vagally-mediated [23].

The i.p. or i.c.v. administration of L-Arg significantly reduced food intake in rats. These findings raise the possibility that a post-absorptive mechanism may be involved in mediating the anorectic effects of L-Arg. Jordi et al. [23] suggested that the anorectic effect of L-Arg is mediated centrally via the area postrema (AP) in the brain stem. Oral administration of L-Arg solution increased c-fos positive cells in AP, and the anorectic effect of L-Arg was abolished in animals that had undergone AP lesioning surgery, although, as mentioned earlier, these studies were performed using the non-salt, basic L-Arg solution. We have previously reported an increase in c-fos-positive cells in the AP after oral L-cysteine administration, suggesting that there may be similar mechanisms by which amino acids influence food intake [29]. Branched-chain amino acids have also been shown to reduce food intake in rodents centrally [50]. Recent studies suggest that a member of the soluble carrier family of proteins, SLC38A9, may play a role in central L-Arg sensing via mammalian target of rapamycin complex 1-dependent mechanisms [51]. Further studies are required to investigate the putative role of post-absorptive mechanisms in the anorectic effects of L-Arg.

In summary, our data further demonstrate the anorectic properties of L-Arg and explore the potential mechanisms involved. The doses of L-Arg administered orally were pharmacological, with the amounts administered acutely being of a similar order of magnitude to the levels that a rodent would consume daily on a 45% high protein diet; however, the present results may also represent pharmacological activation of a physiological nutrient-sensing system. The present chronic administration study suggests that L-Arg may not reduce body weight after repeated dosing, but further work is required to establish the mechanisms involved in mediating the anorectic effects of L-Arg and to explore whether altering the dose and timing of chronic administration might result in significant effects on body weight, and thus suggest therapeutic potential in obesity.

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## Conflict of Interest

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A. Alamshah designed and performed the experiments, analysed the data and wrote the manuscript. A. K. M. designed and performed the experiments and analysed the data. E. S., J. K-J., H. C. O. and I. R. T. performed experiments. A. A., A. M., K. B., R. F., G. H., M. N. and W. C. assisted with experiments. A. L. and H. M. C. designed experiments, provided material support and helped edit the manuscript. S. R. B. provided material and technical support. K. G. M. wrote the manuscript and was responsible for study concept, design and analysis.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**File S1.** L-Arginine promotes gut hormone release and reduces food intake in rodents.

**Table S1.** Feeding studies conditions. Table details the experimental conditions including species, phase of the study, feeding state of the cohort, route of administration and the dose of L-Arg used for each of the feeding studies described in the manuscript. The Feeding study (figure) column refers to the figure number showing the results for each study in the main manuscript. \*All the doses presented in mmol/kg with exception of the ICV study where the dose is given in  $\mu$ mol.

**Figure S1.** The effects of oral administration of L-Arg on behaviour in fasted rats during the light phase. The effect of OG of water (control) or 16 mmol/kg L-Arg in overnight fasted male rats on feeding, locomotion, grooming, head down, pica and resting behaviours compared to control group. Data represented as median (interquartile range) for each observation. n = 12–13.

**Figure S2.** The effect of L-Arg on glucose homeostasis in GPRC6a-KO mice. Glucose tolerance test (GTT) in female WT and GPRC6a-KO mice (A) and the area under the curve for each treatment (B). Mice were fasted overnight and received an

intraperitoneal injection of 20% glucose solution (2 g/kg body weight) followed by an immediate OG of 4 mmol/kg L-Arg. Data is presented as mean  $\pm$  SEM.  $n = 6$  per group.  $*P < 0.05$ ,  $***P < 0.001$  WT-L-Arg vs. WT-L-saline;  $###P < 0.001$  vs. GPRC6a-KO-L-Arg vs. GPRC6a-WT.

**Figure S3.** The effect of GPRC6A on GLP-1 and PYY release from primary murine colonic epithelium. The effect of L-Arg on GLP-1 (A) and PYY (B) release from WT and GPRC6A-KO primary mice colonic L-cells incubated with 100 mM L-Arg for 2 h. The release is shown as percentage of total hormone contained for each well in the experiment. Data is presented as mean  $\pm$  SEM.  $n = 6$  plates, from 6 mice.  $*p < 0.05$ ,  $***p < 0.001$  vs. control.

**Figure S4.** The effect of GLP-1 and Y2 receptors antagonism on the effects of exogenous exendin-4 and PYY on food intake in mice. The effect of IP administration of 400 nmol/kg exendin 9-39 on the anorectic effect of 1 nmol/kg exogenous exendin-4 in fasted mice at 0–1 h post administration ( $n = 10$ ) (A). The effect of IP administration of 5.26  $\mu$ mol/kg BIIE0246 on the anorectic effect of 25 nmol/kg PYY(3-36) in fasted mice at 0–1 h post administration ( $n = 10$ ) (B). Data is presented as mean  $\pm$  SEM. (A):  $**P < 0.01$  vs. saline control,  $###P < 0.001$  vs. exendin 9-39,  $$$$P < 0.01$  vs. exendin-4; (B):  $***P < 0.001$  vs vehicle control,  $###P < 0.001$  vs. BIIE0246,  $$$$P < 0.01$  vs. PYY(3-36).

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